Efficiency of different chitosan and its derivative nanoparticles for delivery of recombinant plasmid of Tibetan pig interleukin-7 gene and regulation effect in vivo on immune responses of mice

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ABSTRACT

Interleukin-7 is a key cytokine to regulate survival and growth of both B and T lymphopoiesis. For sake of developing safe and effective technique to deliver and express animal immune gene *in vivo* to enhance the comprehensive immunity of animal against infectious disease, the interleukin-7 gene of Tibetan pig was firstly cloned and reconstructed into eukaryotic expression plasmid,VR1020. The recombinant plasmid, VRTPIL-7, was expressed in HEK293 cells *in vitro* to confirm its bioactivity on lymphocytes of pig. Then VRTPIL-7 was respectively packed by polyethylenimine (PEI), chitosan(CS) and its two derivatives, methoxy poly(ethylene glycol) (PEG) and PEI modified CS(CS-PEG-PEI) and PEG-modified galactosylated chitosan (CS-PEG-GAL) via by ionotropic gelation method. Their nanoparticle sizes, dispersivity and zeta potentials were analyzed with a transmission electronic microscope and a Zetasizer3000 HS/IHPL instrument (Malvern Instruments Ltd., Malvern, UK). Subsequently, nanoparticles of VRTPIL-7 wrapped with CS, CS-PEG-PEI and CS-PEG-GAL were utilized to intramuscularly inoculate Kunming female mice at the age of 21 days, with a dose at 100μg recombinant plasmid per mice for studying the regulation effect on immunity *in vivo*. Their blood were respectively collected before and after inoculation on 1, 2, 3, 4 and 5 weeks to test the changes of immune cells, IgG□IgG1, IgG2a and the expression level of immune genes by ELISA and quantitative RT-PCR in mice. The results were found that the immune cells numbers in peripheral blood, immunoglobulin (IgG, IgG1, IgG2a) content was significantly increased in the serum of treated mice compared with the control group($P<0.05$). Flow cytometry and q-PCR analysis results showed that the $CD4^+$ and $CD8^+$ T cell numbers and mRNA level of TLR1, TLR4 TLR6, TLR9, TGF-β, IL-1, IL-4, IL-6 andIL-23 were significantly higher in the treated group than those of the control($P<0.05$). These results also indicated that the VTPIL-7 wrapped with CS-PEG-PEI and CS-PEG-GAL induced better innate and adaptive specific humoral and cellular immunity than chitosan, and improved the immune protection and disease-resistant ability of mice against challenge, which implying CS-PEG-PEI and CS-PEG-GAL could be promising practicable package molecules to deliver and express immune gene for prevention and control of animal infectious diseases.

Keywords: **c**hitosan and its derivatives, nanoparticle package and delivery, gene expression, pig interleukin-7, immunity

1. Introduction

Interleukin-7 (IL-7) plays an important role in the immune system which regulates the survival and development of myeloid and lymphoid cells. It was firstly discovered in 1980s as a factor that promotes the growth of pre-B cells in bone marrow culture (Namen AE et al,1988; Kittipatarin C et al. 2007).

Evidences show that Tibetan pigs have greater resistance than domestic pigs against infectious diseases, like CSFV, PRRSV, PCV2, Haemophilus parasuis, etc. Tibetan pig was originally and exclusively found on the Tibetan plateau, a harsh and natural environment with an average altitude approximate of 4 kilometers above sea level. Tibetan pig shows distinct evolutionary scenarios compared with other pigs throughout natural selection. To further explore and clarify the immune characteristics of Tibetan pig is of great importance to understand its unique genetic characteristics for pig breeding, vaccination and prevention of diseases.

So we are interested in how IL-7 exerts unique bioactivity in animal, and try to figure out the expression effect of Tibetan IL-7 gene *in vivo* on immunity of animal for sake of development of safe and novel immunoadjuvant to promtoe the control of infectious diseases in animal in the future.

2. Materials and methods

2.1 Sample and animals.

Lymphocytes were purified from the blood of healthy Tibetan pig provided by Sichuan Academy of Animal Science according to routine method and cultured in 1640 medium with 5 ug/ml Con A for 48 hours, and the RNA was extracted from the activated leukocytes following the instruction of RNA extraction kit manual of Invitrogen.

2.2 TPIL-7 gene cloning and sequencing.

RT-PCR is used for TPIL-7 gene cloning into pMD®19-T vector (Takara), and then transformed into DH5α competent cells. Primers were designed by Primer 5.0 with *BamH I* and *Bgl II* sites according to conserved ORF

sequence of IL-7 gene of Duroc□Landrace and other mammalians from NCBI/GenBank. TPIL-7-F: 5'-*ACCACGCCCGCCTCCCGCAGACCATGTTC*-3', TPIL-7-R: 5'-*GAATATTGAGAGATACGGAGTGGCAA*-3'. Plasmids were assessed for conformity by PCR and digested by *BamH I* and *Bgl II*. Subsequently, the pMD®19-T/IL-7 plasmids were sent to BGI biological company for sequencing. TPIL-7 sequence was aligned with domestic pigs and other mammalians by NCBI/Blast.

Plasmid VR1020 is a eukaryotic expression vector (Vical Company of America). We cloned the TPIL-7 into VR1020 to constructed VTPIL-7 to get secreted protein for *in vitro* and *in vivo* bioactivity test using the same fragment and methods as above mentioned.

2.3 Preparation of recombinant VTPIL-7 and copolymer/DNA nanoparticles

A recombinant DH5a *E. coli*, containing VTPIL-7 or VR1020 plasmid, was inoculated in LB broth with kanamycin (100 mg/ml) at 37□, 200rpm overnight. Bacterial cells were pelleted by centrifugation and plasmid was extracted following the spermine precipitation method described as Jason. Then the plasmid was resuspended in sterile water and stored at -20 □ until use.

Copolymers as CS, CS-PEG–PEI (Yu YY, et al. 2010), CS-PEG-LAC (Song B, et al.2009) and PEI (MW: 25000)/DNA complexes were prepared following the method of by ionotropic gelation method. Briefly, CS, CS-PEG–PEI and CS-PEG-LAC were diluted separately in CH3COOH/CH3COONa (pH 5.5) containing appropriate amount of triphosphate and heated at 65□ for 10 min with mild magnetic stirring. After that, an appropriate amount of polymer solution was added to the solution of plasmid DNA and the solution was mixed then left for 5 min. The zeta potential and average diameter of the polymeric micelles were characterized by Zetasizer 3000 HS/IHPL (Malvern Instruments Ltd., Malvern, UK).

2.4 Transfection and proliferation activity.

HEK293 cells were seeded in 12-well plates (1.0×10^5 cells/well), respectively. The cells were incubated in Dulbecco's modified Eagle medium (DMEM, Invitrogen Corporation.). The complexes of nanoparticles enwrapped with CS, CS-PEG–PEI, CS-PEG-LAC and PEI each containing 3 μg of DNA were added into the wells, respectively, then the cells were incubated with the complexes at 37□in a 5% carbon dioxide humidified atmosphere.

The bioactivity of the TPIL-7 protein was measured by its ability to provoke the proliferation of pig lymphoblast stimulated with Concanavalin A (Con A) through CCK8 colorimetry. Lymphocyte Separation Medium was used to separate of pig peripheral blood mononuclear immune (PBMI) cells for *in vitro* bioactivity test.

2.5 Animal vaccination

Forty 4-week-old female Kunming mice (purchased from the Animal Center of West China Center of Medical Sciences, Sichuan University) were randomly assigned to four groups (A1, A2, A3, C), 10 mice in each group. All the mice were muscularly injected with 6 pmol of 100μg plasmid DNA encoding TPIL-7 per mouse in the left and right quadriceps on day 0. Mice in group A1 were injected with CS/VTPIL-7, A2 were injected with CS-PEI-PEG/VTPIL-7, A3 were injected with CS-PEI-LAC/VTPIL-7 and group C were injected with CS-VR1020 as a negative control. Peripheral blood samples were collected from the tail vein of mice on days 0, 7, 14, 21, 28 and 35 after immunization.

2.6 Bioactivity assay of TPIL-7 *in vivo.*

2.6.1 Changes of cells in peripheral blood.

Peripheral blood samples were used EDTA for anticoagulation, double diluted with normal saline, then analyzed by blood testing instrument: MIND-RAY BC-3000 intelligent automatic blood cell analyzer. Blood test include red blood cells, white blood cells, hemoglobin and platelet count and so on.

2.6.2 Assay of CD3, CD4 and CD8 positive T cells by FCM.

Monoclonal antibody for FCM including Anti-Mouse CD3e FITC (0.5ug/Test), Anti-Mouse CD8a PE (0.25ug/Test), and Anti-Mouse CD4 PerCP-Cy5.5 (0.25ug/Test) for each Test contain 50μl peripheral blood (monoclonal antibody were purchased from eBioscience Company, San Diego, USA.,). FACSAria BD and FACSDiVa BD were used for FCM analysis(BD Biosciences, USA). The method is the same as manual.

2.6.3 Measurement of IgG, IgG1, IgG2a.

Mouse IgG, IgG1, IgG2a measurement ELISA kits were purchased from Bethyl Laboratory, Inc., America. The sandwich ELISA was conducted according to the manufacturer's protocols.

2.6.4 Analysis of TLRs and Cytokines.

According to reports in GenBank, 10 pairs of specific primers were designed and synthesized for mouse β-actin, TRL1, TLR4, TLR6, TLR9, TGF, IL-1, IL-4, IL-6 and IL-23 cDNA sequence.

Quantitative real-time PCR was performed to analyze the gene expression of TRL1, TLR4, TLR6, TLR9, TGF-β, IL-1, IL-4, IL-6 and IL-23 and β-actin as reference gene. The relative expression of cytokine mRNA was calculated relative to the mean expression of the cytokines/TLRs mRNA in the control pigs using the geometric means method and the formula as : relative level = $2^{-\Delta\Delta Ct}$. The ΔΔCt was calculated by the formula: $\Delta Ct_{\text{immunized group}} - \Delta Ct_{\text{control group}} = (Ct_{\text{cytokine/TLR}} - Ct_{\text{reference gene}})_{\text{immunized group}} - (Ct_{\text{cytokine/TLR}} - Ct_{\text{reference gene}})_{\text{control group}}$, where $CT_{\text{cytokine/TLR}}$ is the mean Ct value of triplicates and each $CT_{\text{reference gene}}$ is the mean Ct value of triplicates from the reference gene. Significant differences between the experimental groups were calculated for each cytokine using a Mann–Whitney rank sum test on the relative cytokines/TLRs levels.

2.7 Statistical analysis.

Statistical significance was analyzed using Student's t-test. Differences between experimental groups were considered significant when P-value was less than 0.05.

3 Results

3.1 TPIL-7 gene cloning and sequencing.

TPIL-7 gene cDNA is 571 bp of open reading frame (ORF), encoding 528 bp, and 176 amino acids. TPIL-7 cDNA, alignment with the NCBI pig IL-7 cDNA, have more than 92% homology, and the cDNA sequence of TPIL-7 was submit to GenBank, Sequence ID: gb|KF246514.1|.

3.2 Sizes and zeta potential of nanoparticles.

The cationic polymers as CS, modified-CS and PEI have been considered as excellent candidates for preparation of nanoparticles. Nanoparticles with specific size and positive charge on surface are necessary for endocytosis. The diameter and zeta potentials of the VPIL-7 nanoparticels are revealed in table 1.

Table 1. The diameter and zeta potential of package nanoparticles

	Size (d.nm)		Zeta Potential (mV)	
Sample	X	SD(±)	X	SD(±)
CS/DNA	522.2	5.15	+40.5	1.6
PEI/DNA	341	5.79	+45.5	2.04
CS-PEG-PEI/DNA	169.7	4.07	+43.9	1.11
CS-PEG-LAC/DNA	247.1	18.78	+40.2	0.78

3.3 Proliferation of pig lymphoblasts *in vitro*.

The results showed the significant proliferation of pig lymphoblasts were achieved by addition of the culture supernatant of TPIL-7 gene transfected cells in comparison with control group (Figure 1). Supernatants of CS-PEG–PEI and CS-PEG-LAC groups manifested the strongest proliferative effect on pig lymphoblasts, indicating that the TPIL-7 recombinant palsmid packed with modified chitosans can promote the expression TPIL-7 protein to stimulate better lymphocyte proliferation of pigs (P<0.05).

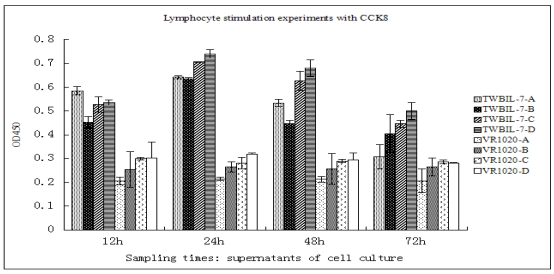

Figure 1. The proliferation results of pig lymphoblasts. A：CS B：PEI C：CS-PEG-Lac D：CS-PEI-PEG

3.4 Promotion of immunity of mice *in vivo*.

3.4.1 Blood immunological change of mice.

During 0-35 days postinoculation, the growth rate of body weight of the treated groups was significantly higher than control group (P<0.05), but after 21 days of it gradually slowed. There were no significant differences among the groups treated with VTPIL-7 nanoparticles (P>0.05) (Figure 2), and the leukocytes of the treated mice significantly increased compared to the control group(P<0.05).

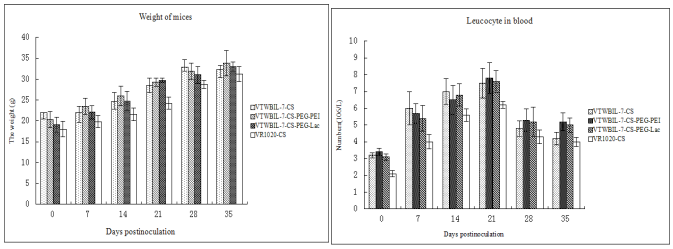

Figure 2. Changes of body weight and immune cells in the blood of the experimental mice.

3.4.2 CD4 and CD8 positive T cells.

The $CD4^+$ and $CD8^+$ T lymphocyte were both significantly increased (P<0.05) in peripheral blood of the treated mice, but the VTPIL-7 packed with modified chitosans resulted in higher increase of the T cells (P<0.05) than the control group from 21 to 35 days post inoculation (Figure 3).

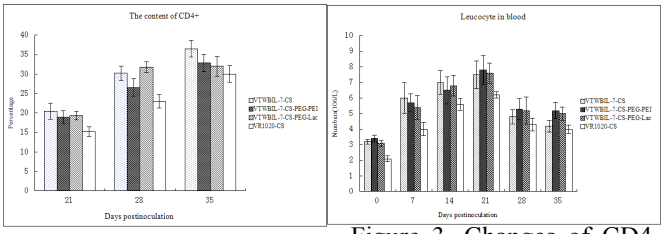

Figure 3. Changes of CD4, CD8 positive T cells in the blood of the experimental mice.

3.4.3 Increase of IgG, IgG1 and IgG2a.

Figure 4 showed that the IgG, IgG1 and IgG2a significantly increased in the serum of treated groups than the control (P<0.05). The ratio of IgG1/IgG2a maintained at about 1.2. This shows that the mice are more likely to induce humoral immune response.

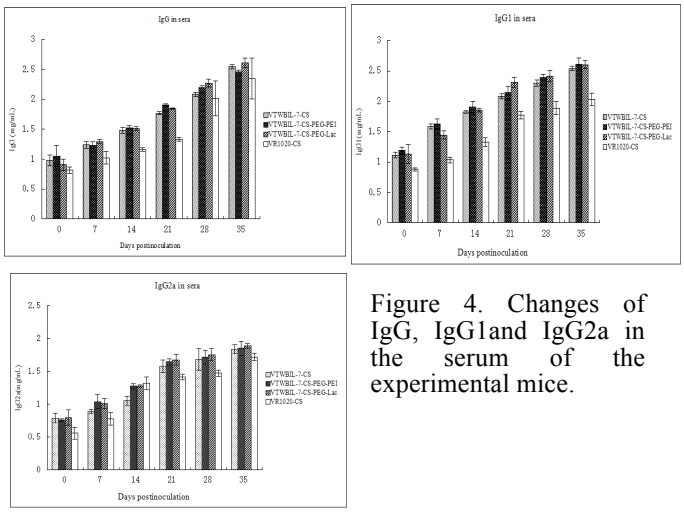

Figure 4. Changes of IgG, IgG1and IgG2a in the serum of the experimental mice.

3.4.4 Enhancement of TLRs gene expression.

The results showed that TLRs gene expression were increased significantly during 0-35 days as showed in figure 5 (P<0.05). TLR4 and TLR9 increased significantly than other TLRs in the treated mice. But TLR6 showed more cascade effect which could be six times higher the data before inoculation, and CS-PEG-PEI group demonstrated obviously better effect than other treated groups (Figure 5).

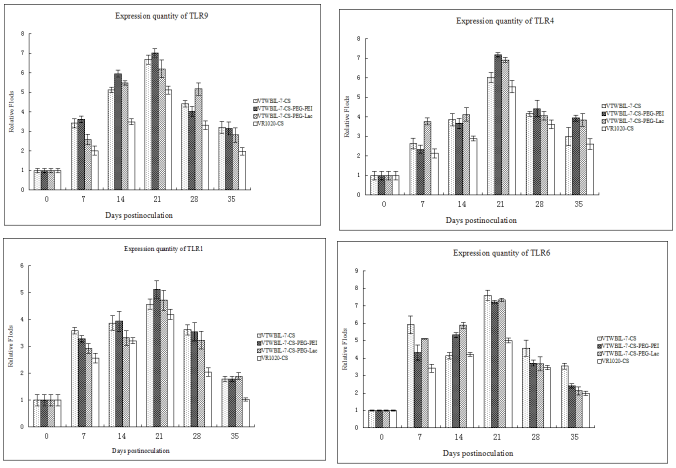

Figure 5. Change of the expression of TLR1, TLR4, TLR6 and TLR9 gene of the experimental mice

3.4.5 Elevation of cytokines gene expression.

Transcription levels of TGF-β, IL-1, IL-4, IL-6 and IL-23 genes were significantly increased in the three treated mice in comparison with those of the control during 35 days period ($P<0.05$), and CS-PEG-PEI group showed higher increases of these genes expression than the other treated mice ($P<0.05$) (Figure 6).

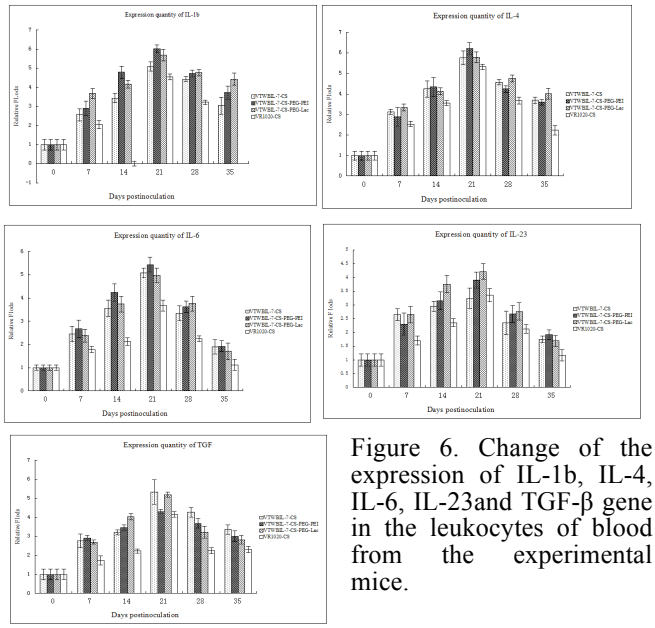

Figure 6. Change of the expression of IL-1b, IL-4, IL-6, IL-23and TGF-β gene in the leukocytes of blood from the experimental mice.

4. Discussion and Conclusion

Nanoparticles with positive charge can help the recombinant plasmid penetrate the cell membrane of transfected cells. And the average particle size ranged from 30nm to 400nm, which were qualified for efficient transfection. The supernatant of HEK293 cells was collected to stimulate lymphoblast of pigs to confirm the biological activities of TPIL-7. These results showed that the TPIL-7 gene was successfully expressed in the transfected HEK293 cells, and the supernatant had dramatically biological activity to promote the proliferation of pig lymphoblast *in vitro*.

The results were found that the immune cells numbers in peripheral blood, immunoglobulin (IgG, IgG1, IgG2a) content was significantly increased in the serum of treated mice compared with the control group ($P<0.05$). Flow cytometry and q-PCR analysis results show that the $CD4^+$ and $CD8^+$ T cell numbers and gene expression of TLR1, TLR4 TLR6, TLR9, IL-1, IL-4, IL-6, IL-23 and TGF-β were significantly higher in the treated group than those of the control group($P<0.05$). These results indicate that the VTPIL-7 wrapped with chitosan and chitosan derivative can significantly improve the innate immunity, humoral and cellular immunity, which could lead to stronger immune protection and disease-resistant ability of animals.

The cationic polymers as CS, modified-CS and PEI have been considered as excellent candidates for preparation of nanoparticles. Nanoparticles with specific size and positive charge on surface are necessary for endocytosis. Researches showed that many of proteins, drugs and DNA complexes were sensitive to bonding with cationic biomaterials delivery system; therefore, multi-cationic PEI was introduced onto CS to enhance the positive charge of the nanoparticles. The average diameter of CS-PEG-PEI was smaller than packaged with CS which probably due to the increase of DNA binding/condensation capability. The zeta potential of CS-PEG-PEI was in the positive range and it was higher than that of CS due to the introduction of polymeric PEI. As expected, the chitosan derivatives resulted in better expressions of the TRL1, TLR4, TLR6, TLR9, TGF, IL-1, IL-4, IL- 6 and IL-23 genes of the treated mice, indicating that the expression of TPIL-7 gene was promoted by the modified nanoparticle molecular package.

RT-qPCR results showed that the expression of TRLs and cytokine genes were significantly increased in the inoculated mice with modified chitosan VTPIL-7 ($P<0.05$) . These suggested that the TPIL-7 gene wrapped with chitosan derivatives could inspire further development of safe and effective immune adjuvant of animal for the control of infectious diseases in the future.

Reference

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